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Exploring miR-222-3p and miR-16-5p: promising biomarkers for gestational diabetes mellitus diagnosis

Zahraa Abdulhadi Nima [1], **Orass Madhi Shaheed*** [1]

[1] *Department of Medical Microbiology, College of Medicine, University of Al-Qadisiyah, Iraq*

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Abstract

Gestational diabetes mellitus (GDM) is a common complication during pregnancy, associated with various adverse maternal and fetal outcomes. Women with GDM have increased risks of cesarean delivery, pre-eclampsia, and gestational hypertension. Additionally, GDM raises the risk of impaired glucose metabolism, obesity, and cardiovascular diseases. This study enrolled 100 pregnant women under medical supervision at the Maternity and Children's Teaching Hospital in Al-Diwaniyah Governorate from September 5, 2024, to May 28, 2025, including 50 diagnosed with GDM and 50 healthy controls. There was no significant difference in mean age between the groups, although GDM patients tended to be slightly older. Expression levels of miR-222-3p and miR-16-5p were measured by quantitative real-time PCR. The mean Ct value for miR-222-3p was significantly lower in GDM patients (26.70) compared to controls (28.79), indicating higher expression in the patient group. Conversely, expression of miR-16-5p was reduced in GDM patients relative to controls. Our findings suggest that elevated miR-222-3p and decreased miR-16-5p levels in maternal blood may serve as promising biomarkers for early diagnosis and risk assessment of GDM.

Keywords: Gestational Diabetes Mellitus (GDM), Micro-RNAs (miRNAs), Pathophysiology, Impaired glucose metabolism, Gene expression.

1. Introduction

A major health issue affecting pregnant women is gestational diabetes mellitus (GDM) [1]. Chronic insulin resistance related to diabetes usually develops in the second half of pregnancy and is a key feature of the pathophysiology of GDM, which is why diabetes often cannot be diagnosed until the late second trimester or early third trimester [2]. Among the greatest common illnesses caused by metabolism worldwide is this particular condition. Throughout globally, diabetes is currently the third "silent killer" after cardiovascular disease and cancer because of its rising rates of illness and death [3]. GDM-complicated pregnancies are linked to fetal and maternal complications. Premature rupture of membranes (PROM), spontaneous abortions, macro-somia, intrauterine growth restriction (IUGR), Respiratory-Diseases stress, newborn low blood sugar, as well as the necessity of newborn intensive care-unit (NICU) admittance, include all considered bad pregnancy-related outcomes (PRO). Negative perinatal outcomes are highly prevalent in mothers with inadequate glycemic control [4]. The prevalence of GDM varies widely worldwide, ranging from 1% to 28%, depending on screening methods, diagnostic criteria, and population characteristics such as maternal age, socioeconomic status, race or ethnicity, and body composition [5]. The prevalence of GDM is rising, which is a worrying trend. For improved care and early identification, new markers, especially epigenetic ones, are sought [6]. miRNAs (microRNAs) are a novel family of noncoding RNAs, approximately 20–25 nucleotides in length, that play a crucial role in posttranscriptional gene regulation and various cellular functions. Numerous human disorders have been linked to changes in miRNA self-expression, according to profiled gene expression research [7]. The primary objective was to look into the possible involvement of mi. RNAs (miR-16-5p and miR-222-3p) within GDM also how they relate to its clinical characteristics.

2. Material and methods

2.1. The collection of samples

A case-control study was conducted on the following study groups during the period from September 5, 2024, to May 28, 2025. The study was approved by the Ethics Committee of the College of Medicine, University of Al-Qadisiyah and the study protocol was approved by the Ethics Committee of the Iraqi Ministry of Health and Environment. A total of 100 women were enrolled in the study, including 50 women diagnosed with gestational diabetes aged 20 to 42 years, and a healthy control group of 50 women aged between 17 and 35 years, all

* Corresponding author.
E-mail address: orass.shaheed@qu.edu.iq (O. Madhi Shaheed).
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attending the diabetes consultation clinic at the Maternity and Children's Teaching Hospital in Al-Diwaniyah Governorate. An extensive collection of samples was performed on Iraqi patients after a physician made a clinical diagnosis of gestational diabetes by medical history and laboratory examination (HbA1c test) and (fasting test). Five milliliters of venous blood were drawn using sterile, single-use syringes. Two milliliters of blood were placed in an EDTA tube for HbA1c testing, while three milliliters were collected in a gel tube and centrifuged to obtain serum for RNA extraction. Blood samples collected from both groups of women were then used to determine the expression levels of miR-222-3p and miR-16-5p.

2.2. Quantitative Reverse Transcription Real-Time PCR

The Real Time PCR primers for *miR-222-3p and miR-16-5p* were designed in this study by the Primer 3plus, V4, and double checked by the University Code of Student Conduct (UCSC) programs, and with their reference sequences in the National Center for Biotechnology Information (NCBI) database, as follows in Table 1.

2.2.1. Preparing the Primers

Primers were received from the manufacturer in lyophilized condition. After dissolving the lyophilized sample in nuclease-free water according to the manufacturer's instructions, a stock solution with a concentration of 100µM was prepared and stored at -20°C. Diluting 10µL of each primer stock solution in 90µL of nuclease-free water yielded a working solution with a concentration of 10µM, which was maintained at -20°C until use.

2.2.2. Total RNA extraction

A total of five milliliters of blood was taken by venipuncture using disposable syringes from each participant. Using disposable gel tubes and leaving them at room temperature for five minutes to allow them to clot, the blood will be separated by centrifugation for five minutes and using a pipette. In an Eppendorf tube, 250 µl of serum was added to 750µl TRIzol® reagent was added to each tubes, mixed properly, and stored in the refrigerator at -20 °C until examination. Following the separation of blood samples into serum, total RNA was isolated. The total RNA was extracted using TRIzol™ Reagent (ER501-01), according to the manufacturer's instructions.

2.2.3. Assessment of RNA quantity and purity

To determine the quantity and purity of the extracted RNA, the OneC Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) was used according to the manufacturer's instructions. This is a highly reliable and selective method for quantifying low-abundance RNA samples. The concentration of miRNA in all samples is within the range of 75-150 ng/µl, which indicates that miRNA is highly selective for miRNA over other forms of RNA. The absorbance of the samples was measured at two distinct wavelengths to determine RNA purity (260 and 280nm). The presence of an A260/A280 ratio of around 1.90-2.0 suggested that the RNA sample was pure

2.2.4. Quantification of microRNAs

According to the manufacturer's protocol (Table 2), this kit is used to quantify small RNAs (~20 nucleotides or base pairs). The miRNA quantification kit enables rapid detection of all types of small RNA, including microRNAs, as well as single-stranded and double-stranded RNAs. It is highly selective for small RNAs over larger mRNAs and can tolerate contaminants such as salts, solvents, or detergents.

2.2.5. Calculate the gene expression

The expression of *miR-222-3p and miR-16-5p* in patient samples was measured using quantitative real-time PCR (qRT-PCR). *miR-222-3p and miR-16-5p* expression were measured using a relative cycle threshold ($2^{-\Delta\Delta Ct}$) methodology. In addition to healthy control samples, GAPDH was used as an internal control (housekeeping gene).

2.3. Ethical approval

The current study has been managed according to the recommendation guide gained from Medicine-College\ Al-Qadisiyah-University. This work did not include forbidden biological materials or genetically modified organisms. All patients were informed about the research and permitted to obtain a questionnaire and draw blood from them (100 subjects were accepted). The qPCR primers for miRNA-222-3p (MIMAT0000279) and miR-16-5p (MIMAT0000069) were designed using the miRNA Primer Synthesis Program and the ResearchCentral miRNA database to select miRNA sequences. Additionally, Primer3Plus software and the NCBI database were used

Table 1. qPCR primers with their nucleotide sequences and product sizes.

Sequence (5'→3')	Primer Type	Target miRNA
AACAAGAGCTACATCTGGCTACT	Forward (F)	*miR-222-3p*
GTCGTATCCAGTGCAGGGT	Reverse (R)	
AACAAGTAGCAGCACGTAAATATTG	Forward (F)	*miR-16-5p*
GTCGTATCCAGTGCAGGGT	Reverse (R)	

Table 2. The thermal profile of *miRNA 122-5P* gene expression.

Step	Temperature (ºC)	Time (sec.)	Cycles
Enzyme activation	94	30	1
Denaturation	94	5	
Annealing	58	15	40
Extension	72	20	
Dissociation	55 ºC-95 ºC		1

to design the qPCR primers for the housekeeping gene GAPDH (NM_001256799.3) used in this study.

2.4. Statistical analysis

The statistical program for social sciences (SPSS), version 26, was used to describe, analyze, and present the data. For quantitative variables, means and standard deviations (SD) were used. Proportions and frequencies were used for qualitative study variables. The independent T-test was used to compare the two study groups. Two quantitative variables were correlated using the Pearson correlation method. P-values less than 0.05 were considered statistically significant, while those less than or equal to 0.01 were considered highly significant.

3. Results

3.1. Baseline characteristics of subjects

A total of 100 blood samples of pregnant women enrolled as volunteers were collected and divided into two groups: 50 pregnant women with Gestational diabetes mellitus (GDM) and 50 pregnant individual controls who are healthy controls. All of these are categorized according to age, weight, biochemical markers (fasting blood sugar (FBS), glycated hemoglobin (HbA1c). The age range in both patient and healthy groups was 17 to 42 years.

The mean age of GDM patients was 29.16 ± 5.88 years, while that of normal pregnant women was 27.24 ± 6.80 years, with no statistically significant difference between the two groups (P = 0.135). Regarding age groups, the overall distribution included 7 (7.0%) women under 20 years, 51 (51.0%) between 20–29 years, and 42 (42.0%) over 30 years. Among GDM patients, 2 (4.0%) were under 20 years, 25 (50.0%) between 20–29 years, and 23 (46.0%) over 30 years; while in the normal pregnant group, 5 (10.0%) were under 20 years, 26 (52.0%) between 20–29 years, and 19 (38.0%) over 30 years. The distribution difference between the two groups by age group was not statistically significant (P = 0.430), as shown in Table 3.

Results show that the mean age of GDM patients was 29.16 ± 5.88 years, with the highest proportion of patients (25, 50.0%) falling within the 20–29 years age group. In comparison, the control group had a mean age of 27.24 ± 6.80 years. The difference in mean age between the patient and control groups was not statistically significant (P = 0.135).

The present results show a significant difference in the frequency distribution of both groups according to pregnancy number (P=0.001) and weight after pregnancy (P=0.001). However, regarding weight before pregnancy, the present results show that the mean weight before pregnancy was lower in pregnant women with GDM compared to healthy pregnant subjects, but the difference was non-significant (Table 4).

The present results showed that most participants with GDM had 3 or more pregnancies compared to GDM women who had one pregnancy, and the difference was significant (P=0.001).

3.2. Real-time PCR-Quantification of *miRNA222* Expression.

Generally, the mean Ct value for miR-222 cDNA amplification was 26.70 in GDM patients, whereas the control group had a higher mean Ct value of 28.79. Compared to

Table 3. Comparison between patients and control groups in the age group.

Study groups		Age group			Mean ±SD
		< 20 years	20-29 years	≥ 30 years	
Groups	GDM Patients	2 (4.0%)	25 (50.0%)	23 (46.0%)	29.16 ± 5.88
	Control	5 (10.0%)	26 (52.0%)	19 (38.0%)	27.24 ± 6.80
Total		7 (7.0%)	51 (51.0%)	42 (42.0%)	
p-value		0.430 ¥ NS			0.135 † NS

n: Cases-number; **S. D**: Standard-deviation; †: Independent.T.test; ¥: Chi-square-test; **S**: significant at. $P > 0.05$.

Table 4. Frequency distribution of pregnant women with GDM and healthy pregnant subjects according to some features.

Characteristic	GDM patients *n* = 50	Healthy control *n* = 50	
Duration of pregnancy			
< 20 weeks, *n* (%)	12 (24.0%)	17 (34.0%)	
20-29 weeks, *n* (%)	13 (26.0%)	11 (22.0%)	0.543 ¥ NS
≥ 30 weeks, *n* (%)	25 (50.0%)	22 (44.0%)	
Absent, *n* (%)	45 (90.0%)	50 (100.0%)	
Weight before Pregnancy			
Mean ± SD	67.66 ± 6.70	68.31 ± 6.45	0.699 †
Range	53– 96	56– 80	NS
Weight after Pregnancy			
Mean ±SD	76.80 ± 6.58	70.72 ± 8.77	0.001 †
Range	60– 89	60– 90	S

n: Cases-number; **SD**: Standard-deviation; †: Independent.T.test; ¥: Chi-square-test; **S**: significant at. $P > 0.05$.

GDM patients, the control group's average Ct levels were significantly greater. This indicates that miR-222 is more abundantly expressed in the GDM samples. Depicting the initially detected miR-222 in these specimens is crucial. The findings demonstrate that the patient group exhibited a higher copy number of miR-222, reflecting its elevated expression (see Figure 1).

Each quantitative PCR reaction was performed in duplicate for each sample. In each run, samples from both the GDM and control groups were included alongside non-template and no-primer controls. This ensures accurate detection of the originally identified miRNA-222 in the samples. The results demonstrate that miRNA-222 expression was significantly higher in the patient group, as reflected by lower Ct values, indicating a greater copy number (see Table 5).

It encompasses the variance within the means of the Ct levels of the miRNA222 cDNA amplifying replication within every variation, as well as the instance of GAPDH as well as depending upon the normalized version of the Ct results when determining ΔCt. Every investigation grouping's proportional expression of the miRNA222 gene was calculated using the 2-ΔCt data. Each of the controlled specimens having an elevated level of miRNA.222 has served to be a calibrator. In the calculation of the relative expression of the miRNA222 gene in all study groups, the 2-CT results were applied. A calibrator was used, and it was one of the samples from the controls with high expression of miRNA222; the mean of 2-ACt values for the control group was (-3.06) and that for GDM patients was (-0.035). When calculating, the gene expression was significantly higher in the GDM patient group than in the control group.

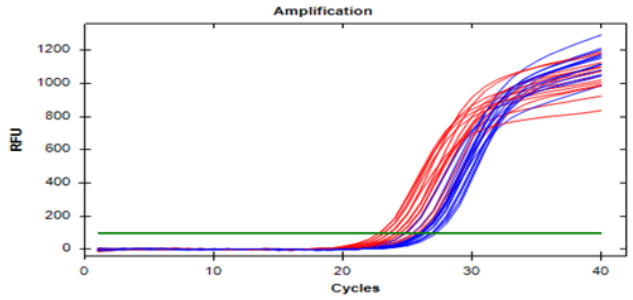

Fig. 1. The real-time PCR amplification plots of *miRNA 222-3p* in patient and control blood samples. The red qPCR plots (patient samples) and the blue qPCR plots (control samples).

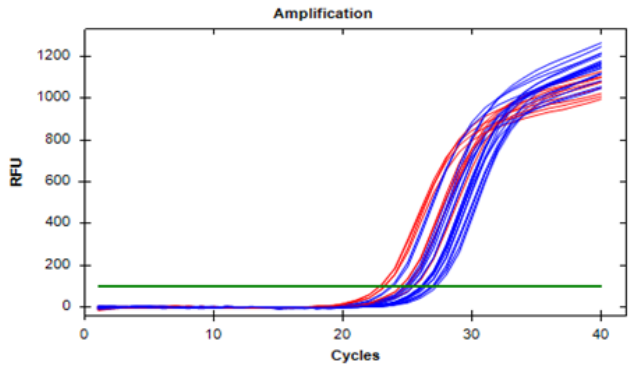

Fig. 2. The real-time PCR amplification plots of *miRNA 16-5p* in patient and control blood samples. The red qPCR plots (patient samples) and the blue qPCR plots (control samples).

3.3. Real-time PCR Quantifications for *miRNA16 Expressing*

The individuals suffering from GDM had an average Ct level of 28.56 for miRNA16 cDNA expression. The overall average Ct levels in the control groups were less compared to those of GDM patients, even though they represented the average (29.98). Representing the initially identified miRNA16 within the specimens is crucial. The findings clearly show that the patient group has the largest copy number of miRNA16, indicating that its expression is reduced. Figure 2 shows the amplification plots and dissociation curves for miRNA-16. The fold change values for the GDM group, as indicated in Table 6, were 0.0027.

This study utilized quantitative RT-PCR to compare *miRNA16* expression between the GDM and control groups. Gene expression changes were assessed using a relative quantification method. This is based on the normalizing for Ct-values of calculation $^{\Delta}$Ct, as well as represents variation among average Ct-values for *miRNA16* cDNA-amplification replica for every instance & *GAPDH* instance. In calculating the relative expression of the miRNA 16 gene expression in all study groups, the 2-4Ct results were applied. A calibrator was used, and it was one of the samples from the controls with high expression of miRNA 16. The mean of 2-4Ct values for the control group was (-2.39) and that for GDM patients was (0.0027). When calculating, the gene expression was significantly higher in the GDM patient group than in the control group.

3.4. Gene Expression Variables within GDM Patients: A ROC Curves Analysis

To evaluate the diagnostic accuracy of miRNA-222 in distinguishing GDM patients from healthy controls, receiver operating characteristic (ROC) analysis was conduc-

Table 5. Comparison of Ct, 2^(-ΔCt), and fold change between GDM patients and healthy controls

Groups	Means Ct for *miRNA-222*	Means Ct for GAP.DH	$^{\Delta}$Ct (Means-Ct for *miRNA-222)*	$2^{-\Delta Ct.}$	Fold of gene expression
GDM patients	26.70	26.69	0.007	-3.06	11.39
Control	28.79	25.72	3.07	-0.035	1.38

Table 6. Comparison of Ct values, 2^(-ΔCt), and fold change between GDM patients and healthy controls.

Groups:	Means-Ct for miRNA16	Means-Ct for GA.PDH	$^{\Delta}$C.t (Means Ct for mi.RNA16)	$2^{-\Delta Ct.}$	Fold of gene expression
GDM patients	28.56	26.69	1.87	-2.39	7.39
Control	29.98	25.72	4.26	0.0027	1.17

ted (Table 7 and Figure 3). Using a miRNA-222 cut-off value greater than 2.75, the area under the curve (AUC) was 0.961 (95% CI: 0.928–0.993, P = 0.001), with sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) all at 96.0%. According to the current findings, the miRNA222 gene is thought to be a highly effective diagnostic marker for differentiating between pregnant women in good health and those with GDM. Additionally, GDM patients may be differentiated from healthy control people using the miRNA16 gene. AUC value represented as 0.952 (95%.CI:0.9031.000., *P* = 0.001), sensitivity represented as 96.0%, specificity value represented as 94.0%, PPV represented as 94.1%, & NPV represented as 95.9% were obtained with an ideal miRNA16 gene cut-off value greater than 2.5. According to the current findings, the miRNA16 gene is thought to be a highly effective diagnostic marker for differentiating between pregnant women in good health and those with GDM.

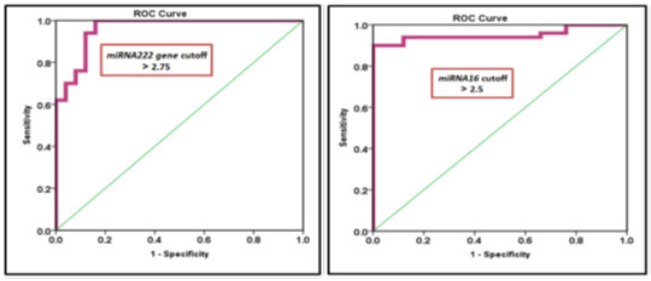

Fig. 3. ROC curve analysis of gene expression parameters in patients with GDM.

4. Discussion

Due mostly to the dearth of efficient diabetes prevention techniques, the incidence of GDM has been rising quickly over the past few decades, raising serious concerns within governing bodies as well as healthcare sector bodies. Based on data, the mean age of pregnant females within the GDM and control-group didn't differ significantly. There was no statistically significant age difference between the two groups, although the GDM patients did have a slightly greater mean age. Considering the history of the past forty years of existence, the mean ages for delivery increased [8]. Understanding the entire range of age-based danger indicators with GDM is crucial. The study suggested aging could be a reflection of β cells' declining capacity to release insulin, which cannot overcome insulin resistance that increases or develops with pregnancy [9]. Gestational diabetes mellitus affects pregnant women of all ages, although the bulk of the cases occur in the 3ed decade of age. Nonetheless, overall is still conflicting data concerning the association involving maternal-age as well as danger of GDM. Understanding the entire range of age-based danger indicators with GDM is crucial. According to one study, the prevalence of GDM rose with increasing-age, maximum within women -ages 35 to 39, as well as subsequently decreased among women-ages 40 to 50. This is in contrast with various research indicating imply the danger of GDM rises exponentially alongside maternal age [10].

The mean age of GDM patients was 29.16 ± 5.88 years, with half of them aged 20–29 years. The control group had a mean age of 27.24 ± 6.80 years. There was no statistically significant difference in mean age between the two groups (P = 0.135). Although they do not prove that maternal-age is a potentially hazardous reason for GDM, the present observations represent a significant discovery. The present results agree with the results of Hussain (2018) [11], which demonstrated that the research individuals' average ages were 28.8±6.1 (range 16-45 years). The specific underlying process of the relationship within maternal-age, as well as GDM, isn't effectively established; this phenomenon is concerning because it appears that older maternal-age isn't the cause of it, as research suggests that more also more young-women will develop GDM. A significant amount of diabetes-related insulin-resistance, circulation-adipokines, as well as markers of inflammation, as well as oxidative-stress may all contribute to this phenomenon [12].

The present finding conflicts with some previous Iraqi studies [13], which reported that the 30–39-year age group is more affected by GDM. Additionally, an earlier research investigation carried out in mainland China found that the adjusted prevalence of GDM peaked in women between the ages of 30 and 34 and then decreased after the age of 35 [14], reported that Women who became pregnant between the ages of less than 23 and 30 years incompatible with young study had the lowest risk of developing the disease compared to women older than 30 years who had primiparas pregnancy, as the incidence of the disease was particularly high. The current study results also disagree with some other Iraqi studies [15], which found that the age indicator was statistically significant for contracting with GDM.

The results indicated that the majority of participants with GDM had three or more pregnancies, which was significantly higher compared to GDM women with only one pregnancy (P = 0.001). These results coincide with Liu et al (2020) [16], who found women who had 2 and 23 pregnancies had 1.29 (95% CI, 1.10–1.51) and 1.89 (95% CI, 1.60–2.23) times higher risk of GDM than women

Table 7. ROC curve analysis of gene-expression parameters in patients with GDM.

Characteristic	*miRNA222 gene*	*miRNA16 gene*
Cutoff value	< 2.75	< 2.5
***P*-value**	0.001	0.001
Sensitivity. %	96.0 %	96.0 %
Specificity %	96.0 %	94.0 %
PPV %	96.0 %	94.1 %
NPV %	96.0 %	95.9 %
AUC (95% CI)	0.961 (0.928- 0.993)	0.952 (0.903- 1.000)

CI: Confidence interval, AUC: Area under the curve.

who had one pregnancy.

A study conducted among 11,205 women found that ≥3 live births increased the risk of GDM in Southeast Asian women [17]. Another study found that multiparty was associated with a higher risk of GDM. Similarly, [18] evaluated that GDM risk increased with the increasing number of previous pregnancies. The mechanism underlying the link between the number of pregnancies and GDM is unclear. During pregnancy, the increased secretion of steroids and peptide hormones leads to a progressive rise in maternal tissue insulin resistance. Although glucose homeostasis is restored to preconception levels shortly after delivery, repeated exposure to these drastic hormonal and metabolic changes may still pathologically perturb glucose metabolism [19].

In this study, quantitative RT-PCR was used to analyze and compare the expression of miRNA-222 between the GDM group and the control group. Alterations within gene-expression were calculated utilizing a relative member quantitative measurement [20].

In this study, quantitative RT-PCR was used to analyze and compare the expression of miRNA16 between the control group and the GDM group. Generally, changes in gene expression were calculated using a relative quantitative method [21].

According to the current findings, it showed a substantial positive association between the miRNA222 gene as well as the miRNA16 gene (r=0.322 and p=0.022), as well as between the miRNA222 gene as well as the level of FBS (r=0.301 and p=0.027). This suggests that there may be a connection within the miRNA as well as the metabolic processes of glucose during gestation, which has been reported to have a major effect on birth weight. These results are consistence [22]. That found the expression of circulating *miR-222-3p* was positively correlated to fasting plasma glucose (FPG) (p<0.001), showing the favorable correlation between the patients' group's miR-222-3p expression and FPG and HbA1c. Additionally, in individuals with GDM, elevated miR-222-3p expression shows a significant correlation with FPG [23]. While the present results show there was significant negative correlation of *miRNA16 gene* (r=-0.327 and p=0.021), these results are inconsistence with the study of Tagoma et al (2018)[24], who showed a significant positive correlation of *miRNA16* among those suffering from pregnancy diabetic-mellitus (P=0.03) revealed that there was a notably favorable connection between miR-16-5p. Gene-expression of miRNAs in mother's WBCs within individuals with concomitant pregnancy-related diabetes-mellitus was assessed for the first time as represented within the present research [24]. The overall area under the curve (AUC) values for miR-222 and miR-16, based on ROC analysis reflecting their prevalence in GDM patients, indicated strong diagnostic potential. Among them, miRNA-222 showed the highest AUC, demonstrating the greatest discriminative ability (P < 0.05). These microRNAs fulfill the criteria for biomarkers and hold promising diagnostic value for GDM detection.

The study suggests that women with Gestational Diabetes Mellitus (GDM) have higher levels of miRNA-222 and lower levels of miRNA-16 in their blood samples. Quantitative RT-PCR was used to analyze the gene expression levels of these two miRNAs, employing the 2^(-ΔCt) method for relative quantification, with GAPDH as the normalization control and a control sample with high miRNA-222 expression serving as the calibrator.

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Conflict of interest
The authors declare no conflict of interest.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
Orass Madhi Shaheed: Research design and supervision; Zahraa Abdulhadi Nima: Perform all laboratory procedures.

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